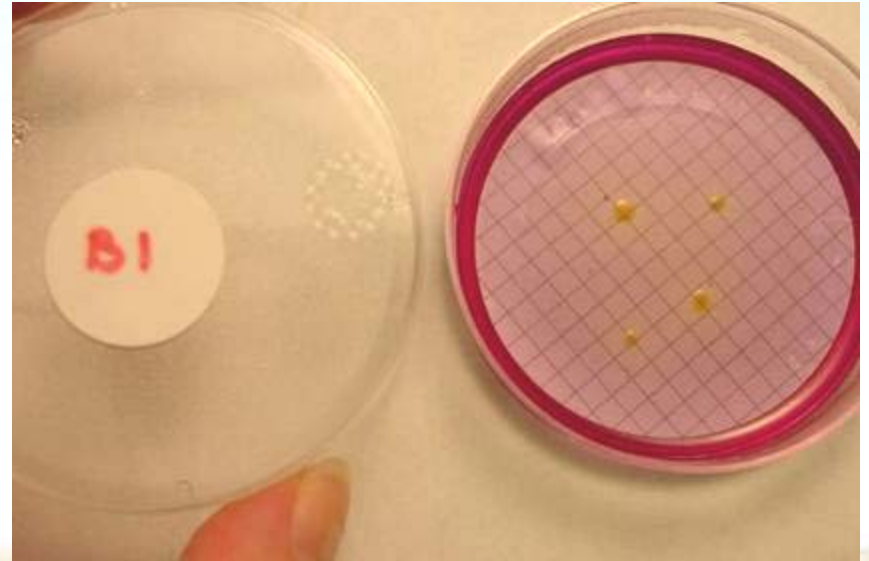
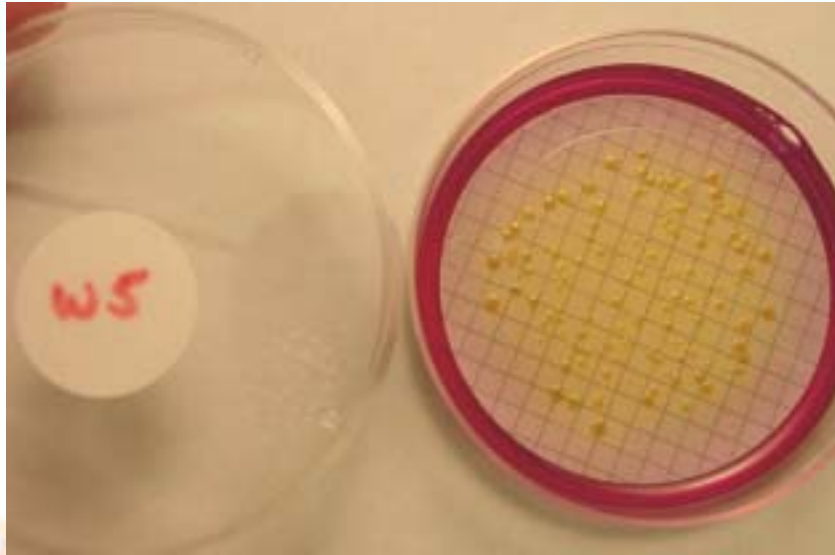


# Microbial Source Tracking in Two Southern Maine Watersheds



Presented at Northeast Shellfish Sanitation Association, May 8, 2003 by  
**Steve Jones, Jackson Estuarine Lab, University of New Hampshire**  
**Fred Dillon, U. Southern Maine-Muskie School of Public Service**  
in close cooperation with

**Kristen Whiting-Grant, Maine Sea Grant**  
**Cayce Dalton, AmeriCorps/Maine Conservation Corps**  
**Michele Dionne, Wells National Estuarine Research Reserve**

# Project Partners

Funded by a grant from:  
**Cooperative Institute for  
Coastal and Estuarine  
Environmental Technology  
(CICEET)**



**In cooperation with  
partners from:**





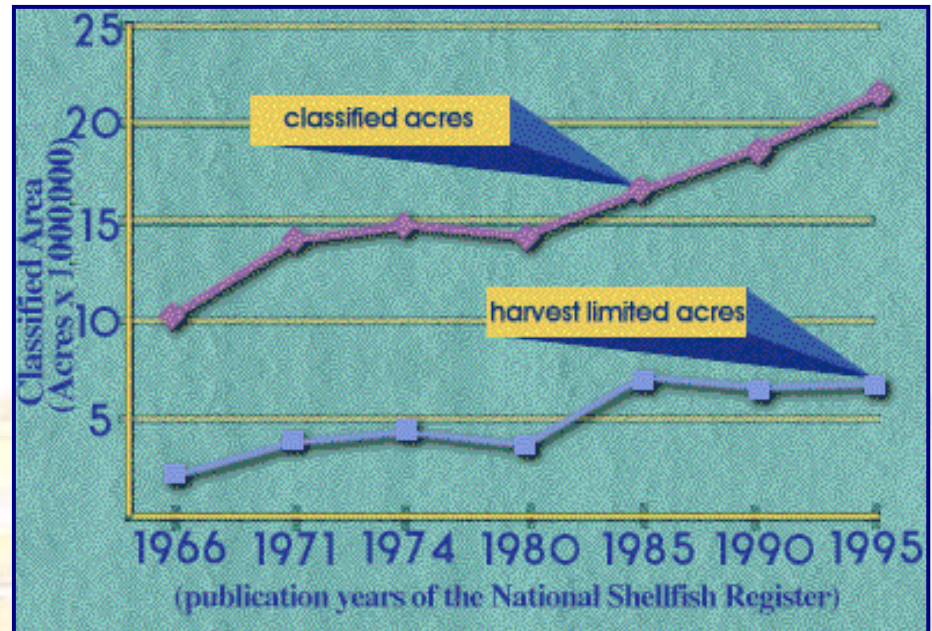


**Problem/Need: History of fecal contamination in Webhannet and Little River estuaries in S ME results in closed clam flats**



# Economic Importance of Shellfish Harvesting Nationally

- In 1995, over 33,000 sq. mi. of marine and estuarine waters in the contiguous United States were classified as shellfish growing waters
- Commercial harvest from these waters totaled 77 million pounds of oysters, clams and mussels worth approximately \$200 million at dockside



Classified growing waters of contiguous U.S.

*6.7 million acres were closed or restricted to shellfish harvesting*

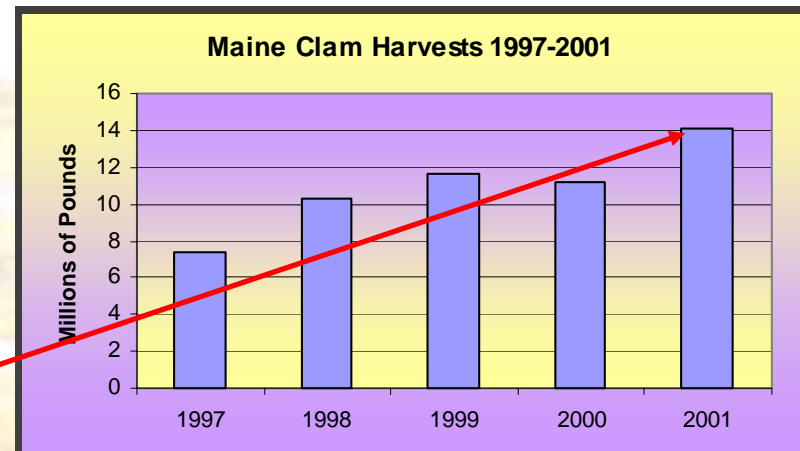
# Economic Importance of Shellfish Harvesting in Maine

According to the Maine DMR, in 2001 there were 1.825 million acres classified as shellfish growing areas

9.1% of this (166,555 acres) was closed to shellfish harvesting mostly due to bacterial contamination

Maine Landing Statistics for 2000			
Species	Metric Tons	Pounds	\$ Value
CLAM, SOFTSHELL	1036.2	2,284,330	\$9,545,531
MUSSEL, BLUE	1287.2	2,837,690	\$1,037,224
OYSTER, EASTERN	11.9	26,306	\$97,594
OYSTER, EUROPEAN FLAT	8.3	18,320	\$67,966
CLAM, QUAHOG	7.5	16,643	\$49,929
CLAM, ATLANTIC JACKKNIFE	0.5	1,193	\$5,140
GRAND TOTALS:	2351.6	5,184,482	\$10,803,384

\*Landings are reported in meat weights (excluding shells)



*The areas currently closed to shellfish harvesting represent approx. \$986,000 in lost revenue*

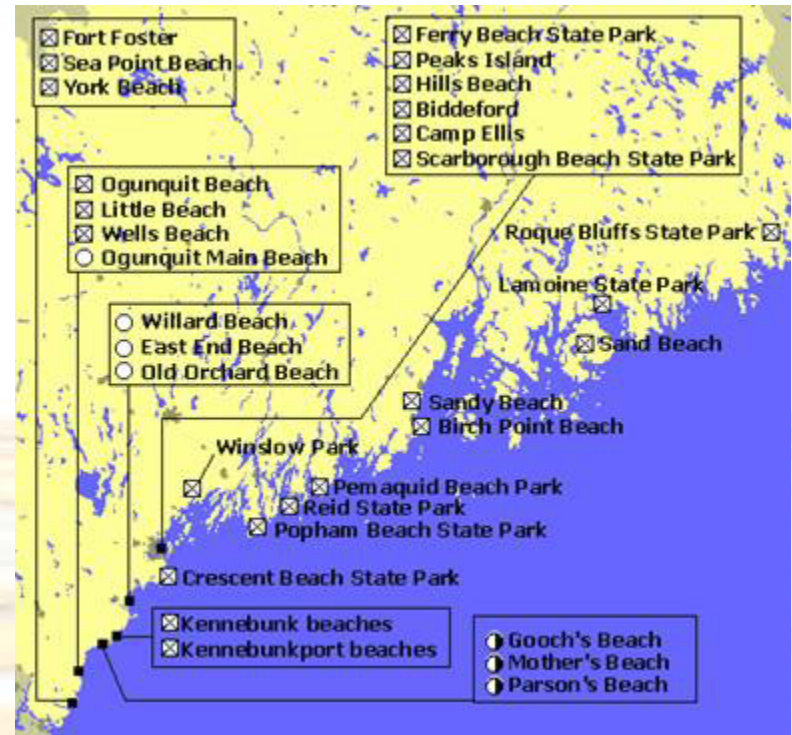


**Potential public health threats at swim beaches (not including the sharks at Wells Beach!)**



# Public Health and Economic Significance of Bathing Beaches

- According to the EPA, a third of all Americans visit coastal areas each year, making a total of 910 million trips while spending about \$44 billion
- Beach tourism is a significant part of these coastal economies
- Many beaches become polluted from storm water runoff and combined sewer overflows

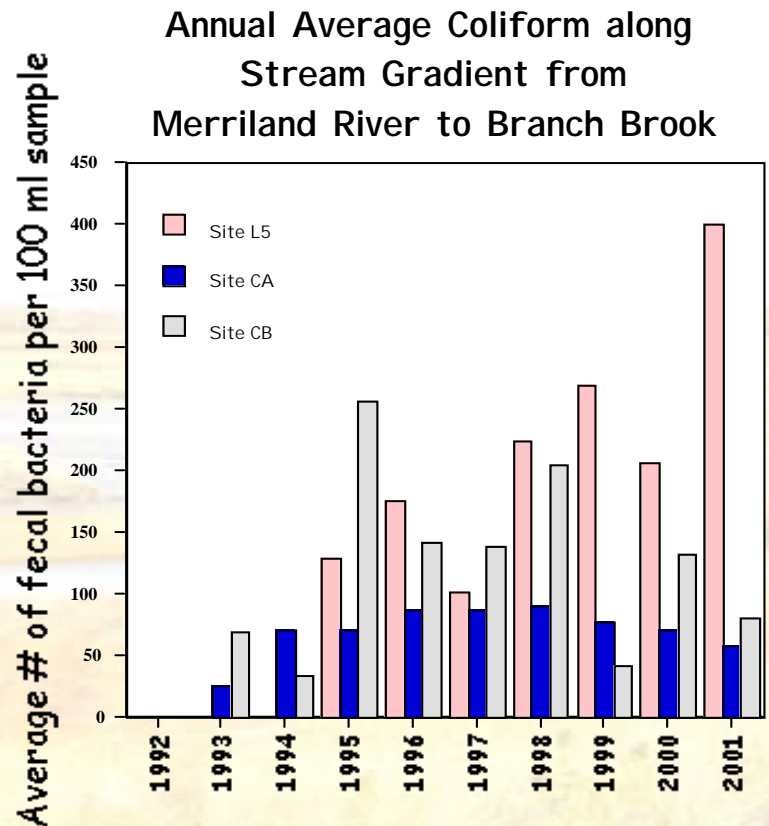


*Despite this public health threat, many polluted beaches are not routinely tested or posted with warning signs*



**In Wells, humans were historically suspected as major contributor: results in extension of sewer in April 2000**

**But still the problem persists...**



***So what ARE the sources?***





## Project Goal 1:

Explore use of technology  
in more closely identifying  
sources of bacterial  
contamination





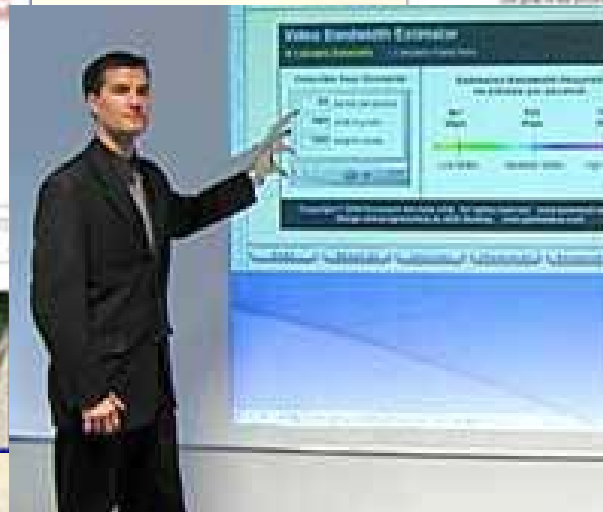
## Project Goal 2:

Use study results to develop targeted management plan for reducing bacterial contamination

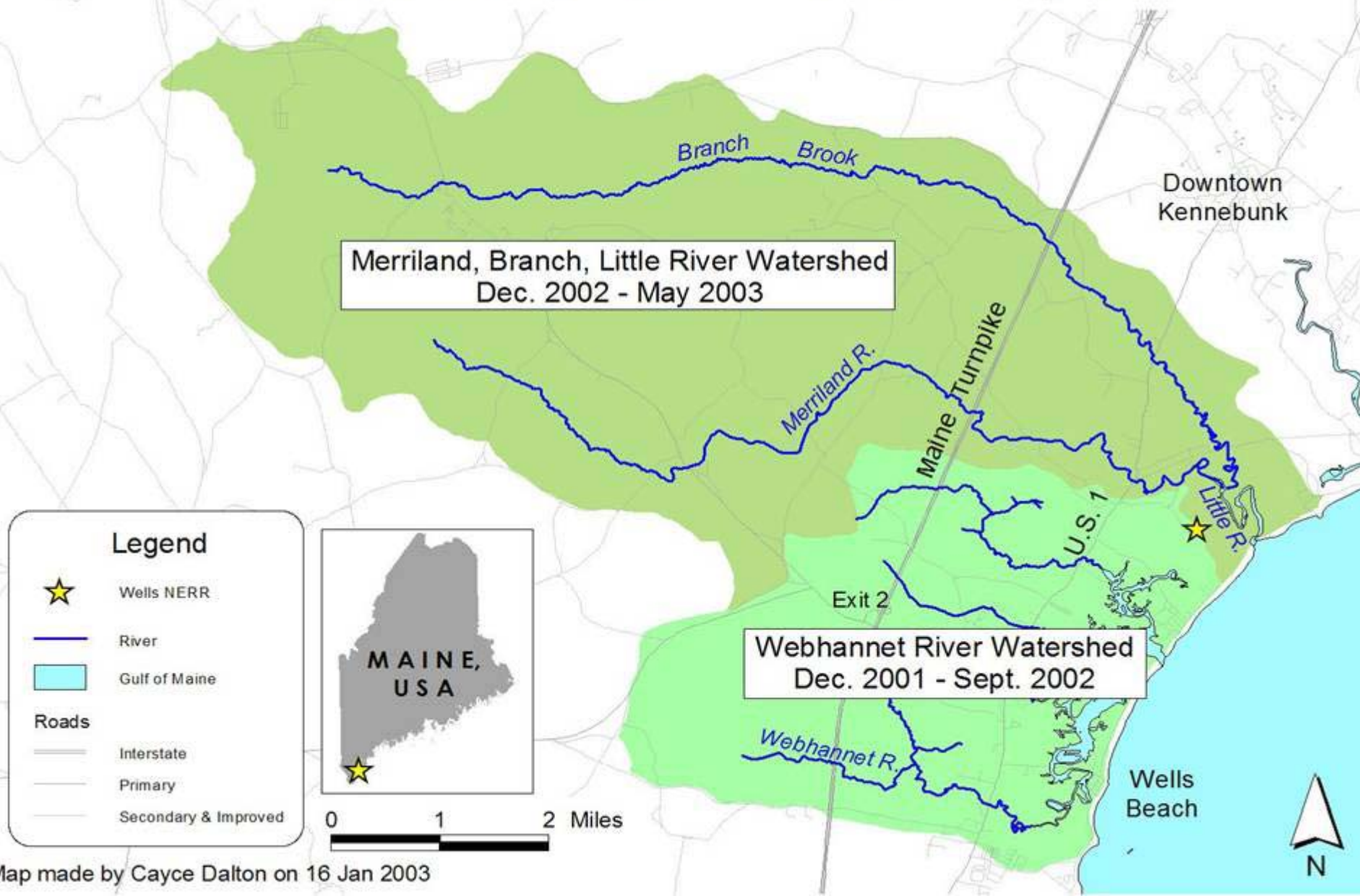
(Hopefully allows for resumption of shellfish harvesting in two coastal watersheds)



### Project Goal 3: Conduct outreach activities to involve citizens and public officials in plan implementation



# Microbial Source Tracking in Two Southern Maine Watersheds: Study Area





# What is Bacterial Pollution?

It indicates presence of fecal matter in water, risk of illness from water contact, and grounds for shellfish bed and swim beach closures.



# Sources of Bacterial Pollution?

- Unmanaged livestock/pet waste
- Leaking sewer pipes/storm



septic systems

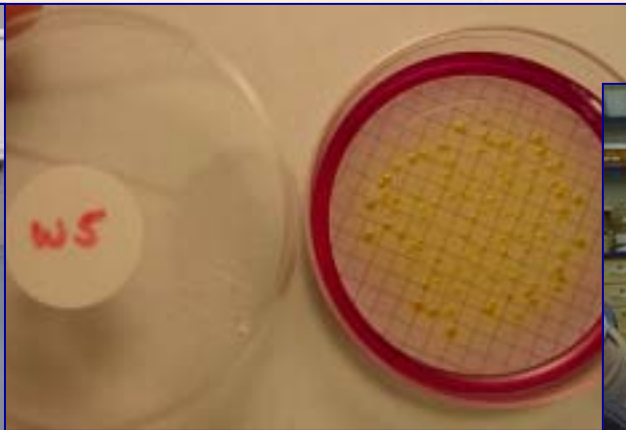


# Importance of Bacteria as Water Quality Indicators

Inexpensive surrogate for hundreds pathogens, because associated with fecal-related pollution.

Countable, not just presence/absence.

Provide regulatory standards for shellfish areas and recreational waters.



# What is Microbial Source Tracking?

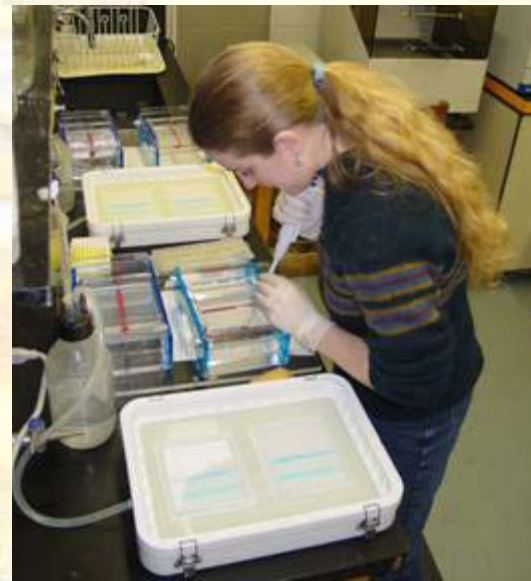
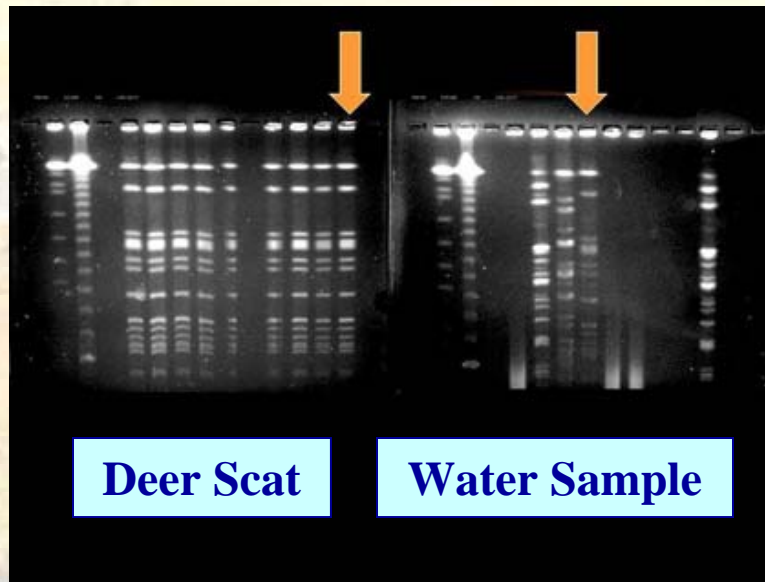
- Group of molecular, genetic and chemical methods to identify specific strains of indicator bacteria or virus in environment
- Unknown strains from environment compared to strains found in host animals
- Close matches are a basis for source identification
- MST is an experimental technique that is gaining attention






# Why Use MST?

- **Addresses biggest weakness of conventional bacterial tests: not source specific.**
- **Knowing sources means corrective measures focused, saving public resources and reducing frustration.**
- **Example: expensive sewer extension in Wells, Maine did not significantly reduce fecal coliform levels in Little River Estuary.**



# Brief Overview of MST Methods



	<i>Library dependent</i>	<i>Library independent</i>
<i>Genotypic</i>	ribotyping bacterial community fingerprinting (t-RFLP) repetitive intergenic DNA sequences (PCR)	F+ coliphage human pathogenic virus detection (PCR or RT-PCR) Bacteroides genotyping (PCR) Enterotoxin biomarkers (PCR)
<i>Phenotypic</i>	multiple antibiotic resistance carbon source profiling	Enterotoxin biomarkers F+ coliphage serotyping IgA Antibodies

Two-way classification of some of the more widely used source tracking methods in terms of their focus on genotypes or phenotypic characteristics and their relative dependence on a background library or database of genotypic or phenotypic characteristics.

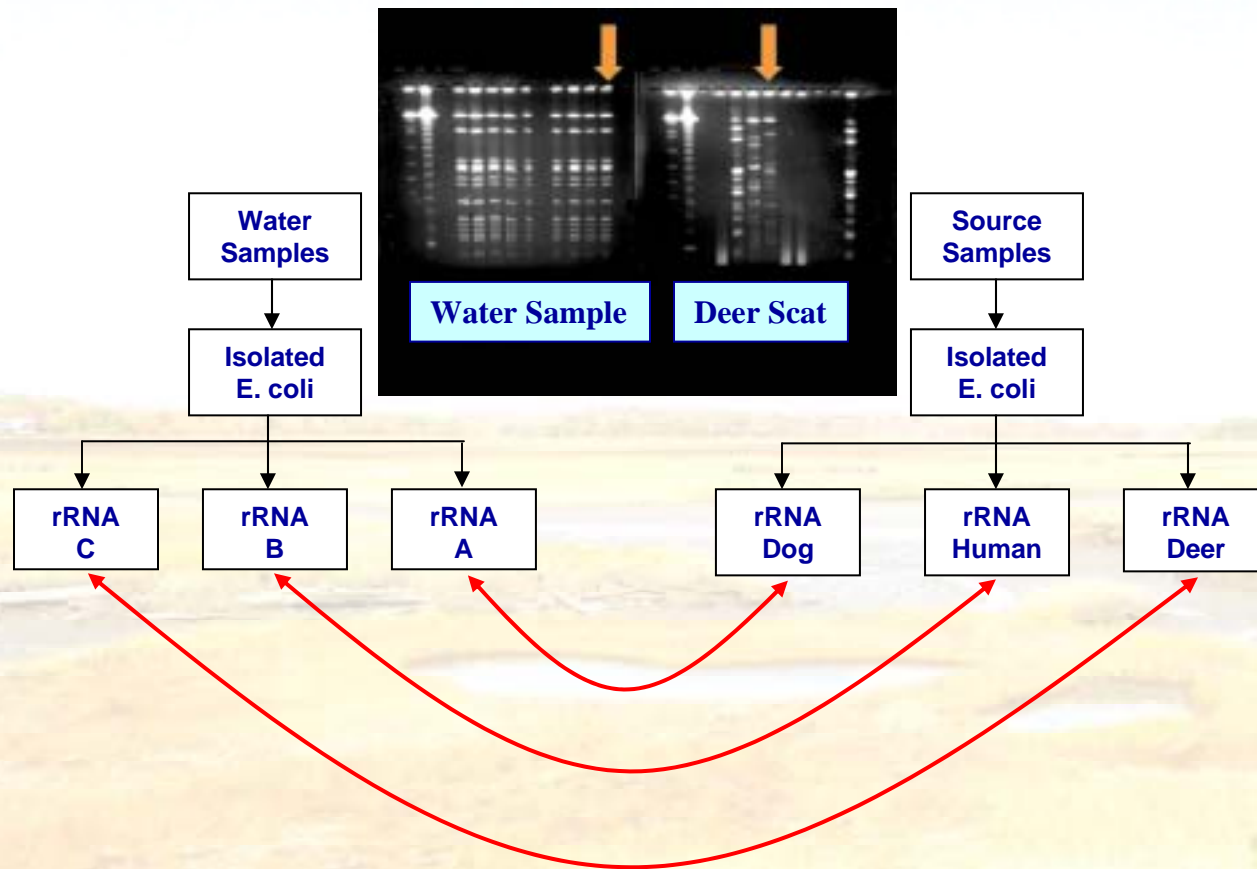
Distinguish among bacterial and/or viral samples based directly on their genetic makeup

Distinguish among samples based on secondary characteristics (such as antibiotic resistance)

(**Libraries** provide basis for comparison with water samples)



# How Ribotyping Works in Brief...

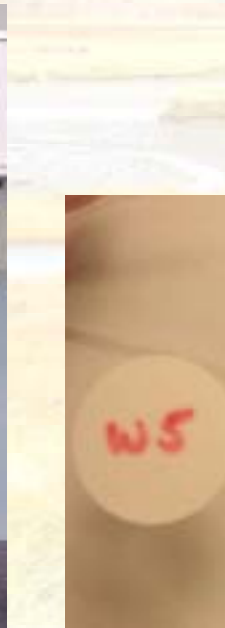


# MST Step 1: Intensive Water Sampling

Sample freshwater tributaries of estuary during winter clamming season.

Half of sampling during post-storm, snowmelt or high flow conditions.

Test is membrane filtration using mTEC + urea for *E. coli*.



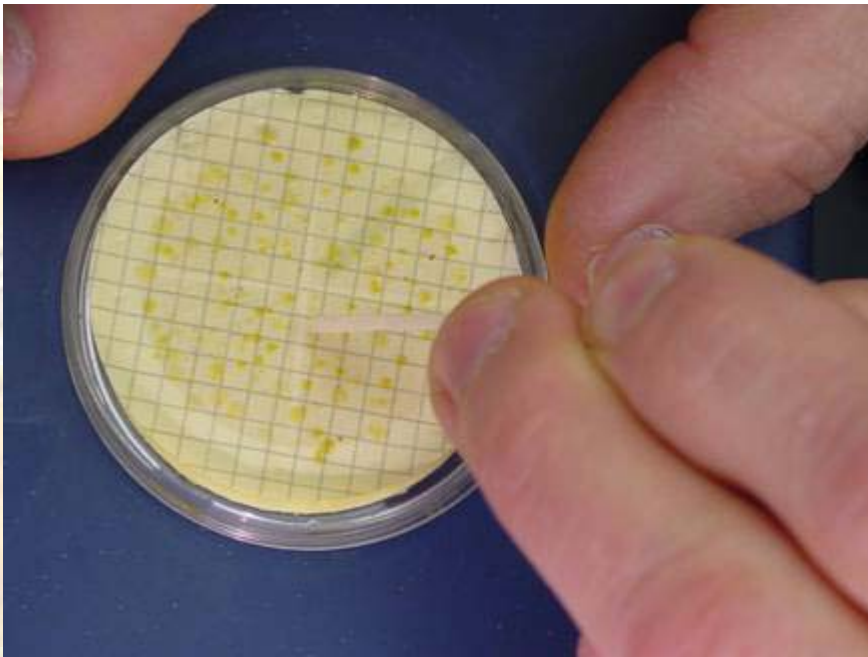


# MST Step 2: Select and Save Bacteria

Samples with high *E. coli* are identified.

10 bacteria are isolated on TSA and refrigerated.

Isolates transported to Jackson Estuarine Lab for ribotyping within about two weeks.



# All samples tested for *E. coli*

<i>E. coli</i> CFU/100mL											
site	12/4/01	1/8/02	1/24/02	1/31/02	2/21/02	3/4/02	3/11/02	4/15/02	5/6/02	5/15/02	5/30/02
W7	91.0	3.3	0.0	1.7	27.0	21.0	1.7	2335.0	8.2	480.0	21.0
W6	67.0	12.5	0.0	3.3	78.0	62.0	77.0	515.0	17.3	300.0	0.0
W5	128.0	11.7	1.7	4.2	80.0	61.0	100.0	1815.0	23.0	380.0	20.0
W4	123.0	34.0	7.5	555.0	17.5	260.0	57.0	98.3	32.0	27.0	25.7
W3	68.0	15.8	4.2	260.0	22.0	200.0	52.5	735.0	22.0	300.0	5.7
W2	78.0	11.7	6.7	360.0	65.0	130.0	88.3	715.0	39.0	200.0	5.7
W1	62.5	39.0	36.0	365.0	41.0	110.0	79.0	490.0	43.0	220.0	54.3
P5	173.3	86.0	62.0	64.0	150.0	60.0	410.0	205.0	16.4	74.0	0.0
P4	27.0	19.0	23.3	19.2	220.0	58.0	605.0	49.5	10.0	152.4	22.9
P3	64.0	10.0	6.8	36.0	160.0	53.0	735.0	60.0	30.0	no data	40.0
P2	70.0	37.0	44.1	36.0	115.0	46.0	795.0	175.0	44.0	150.0	425.7
P1	32.0	28.0	65.0	19.0	185.0	44.0	195.0	110.0	48.0	189.1	85.7
D5	8.0	1.7	0.8	0.8	10.8	2.0	24.0	7.5	21.0	54.0	40.0
D4	14.0	2.5	2.5	9.5	32.0	7.5	16.7	24.0	37.5	51.0	19.0
D3	176.0	24.0	7.5	40.0	150.0	31.0	23.0	215.0	20.0	76.0	20.0
D2	354.5	185.0	27.0	33.0	245.0	22.0	37.0	235.0	17.3	40.0	2.9
D1	148.0	155.0	6.7	29.0	490.0	30.0	21.0	390.0	9.1	28.0	14.3
B4	25.0	1.0	0.0	9.0	160.0	8.0	2.0	387.5	2.4	52.0	7.0
B3	14.0	0.0	0.0	6.0	33.0	9.0	2.0	279.0	1.8	75.0	2.0
B2	1.0	0.0	0.0	15.0	25.0	4.0	0.0	206.0	0.0	80.0	4.5
B1	4.0	5.0	0.0	12.0	14.0	6.0	1.0	43.5	3.6	54.0	50.0



# From samples with high *E. coli*, bacteria are isolated

<i>E. coli</i> CFU/100mL											
site	12/4/01	1/8/02	1/24/02	1/31/02	2/21/02	3/4/02	3/11/02	4/15/02	5/6/02	5/15/02	5/30/02
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W6	67.0	12.5	0.0	3.3	78.0	62.0	77.0	515.0	17.3	300.0	0.0
W5	128.0	11.7	1.7	4.2	80.0	61.0	100.0	1815.0	23.0	380.0	20.0
W4	123.0	34.0	7.5	555.0	17.5	260.0	57.0	98.3	32.0	27.0	25.7
W3	68.0	15.8	4.2	260.0	22.0	200.0	52.5	735.0	22.0	300.0	5.7
W2	78.0	11.7	6.7	360.0	65.0	130.0	88.3	715.0	39.0	200.0	5.7
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P5	173.3	86.0	62.0	64.0	150.0	60.0	410.0	205.0	16.4	74.0	0.0
P4	27.0	19.0	23.3	19.2	220.0	58.0	605.0	49.5	10.0	152.4	22.9
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B2	1.0	0.0	0.0	15.0	25.0	4.0	0.0	206.0	0.0	80.0	4.5
B1	4.0	5.0	0.0	12.0	14.0	6.0	1.0	43.5	3.6	54.0	50.0

# From isolates, a few representative samples are ribotyped

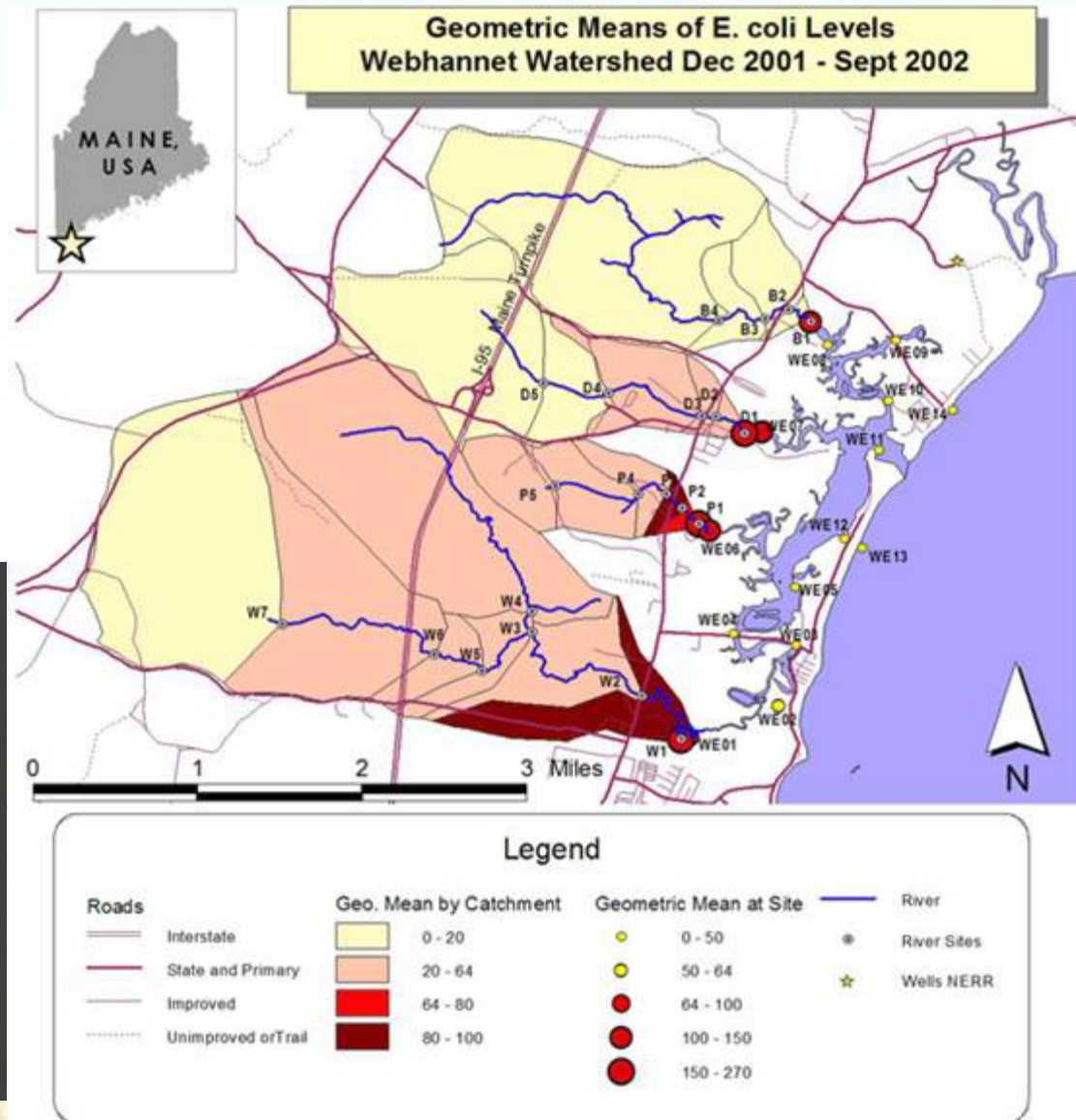
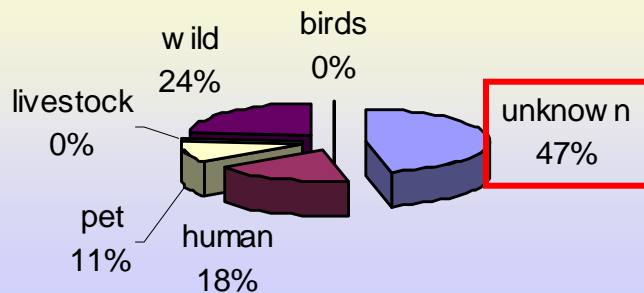
<i>E coli</i> CFU/100mL											
site	12/4/01	1/8/02	1/24/02	1/31/02	2/21/02	3/4/02	3/11/02	4/15/02	5/6/02	5/15/02	5/30/02
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B2	1.0	0.0	0.0	15.0	25.0	4.0	0.0	206.0	0.0	80.0	4.5
B1	4.0	5.0	0.0	12.0	14.0	6.0	1.0	43.5	3.6	54.0	50.0



# What do the results indicate?

- Humans single largest contributor
- Combined wildlife largest overall contributor

Webhannet Source Species Database



# Key Components of Draft Management Plan

## 2.0 DESCRIPTION OF WEBHANNET RIVER WATERSHED

- 2.1 Development and Land Use
- 2.2 Sewering of the Watershed
- 2.3 Shellfish Growing Area Water Quality Monitoring Program
- 2.4 Watershed and Shoreline Surveys

## 3.0 ASSESSMENT OF CURRENT FECAL CONTAMINATION

- 3.1 Sample Site Selection
- 3.2 Sample Collection Procedures and Sample Dates
- 3.3 Defining Wet and Dry Weather Samples
- 3.4 Laboratory Methods & Analytical Procedures
- 3.5 Data Management
- 3.6 Analysis of *E. coli* Data for Water Samples
- 3.7 Selection of Source Species for *E. coli* Reference Libraries
- 3.8 Selection of *E. coli* Isolates for Ribotyping Analysis
- 3.9 Source Species Identification for *E. coli* Isolates from Unknown Water Samples
- 3.10 Source Species Identification in Different Tributaries
- 3.11 Wet Versus Dry Weather Sources
- 3.12 Analysis of Data for Key Shellfish Program Issues

## 4.0 RECOMMENDED MANAGEMENT ACTIONS

- 4.1 Control of Point Sources
- 4.11 Investigation of Wastewater Treatment Infrastructure
- 4.12 Urban Runoff
- 4.13 Overboard Discharges
- 4.2 Control of Nonpoint Sources
- 4.21 Wildlife and Waterfowl Components
- 4.22 Septic Tank Controls and Inspection
- 4.23 Boat Waste
- 4.24 Pets and Pet Waste
- 4.3 Additional (Future) Monitoring

## 5.0 PUBLIC PARTICIPATION / PUBLIC OUTREACH

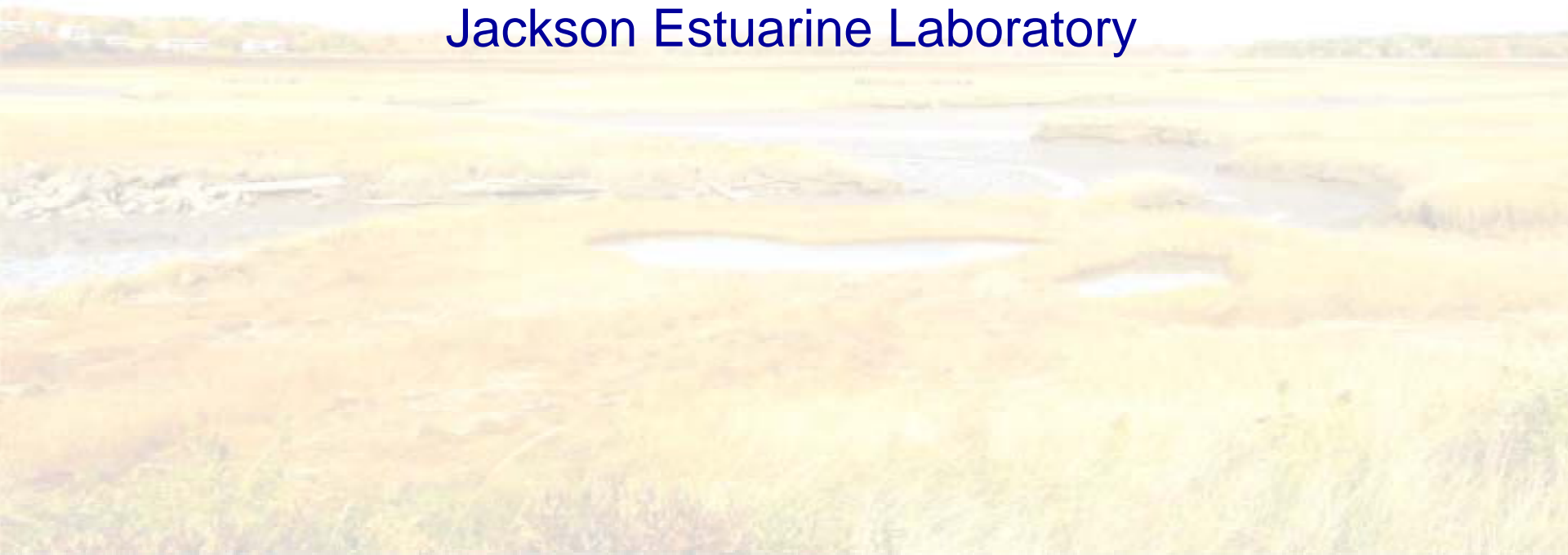
- 5.1 Volunteer recruitment
- 5.2 Web Site Development
- 5.3 Conference / Workshop Presentations
- 5.4 Media Relations: Public Access TV / Radio / Newspaper
- 5.5 Community Outreach for Plan Implementation



# ***RIBOTYPING***

**Steve Jones**

University of New Hampshire  
Jackson Estuarine Laboratory



# NEW TOOL: Microbial Source Tracking

- Identify species that are sources of fecal contamination, not just concentrations of bacteria;
- Multiple applications:
  - identifying the source(s) of indicator bacteria as human, domestic animal, wildlife
  - support sanitary surveys for bathing beaches and shellfish growing areas
- Experimental!



# Microbial Source Tracking: Different Types

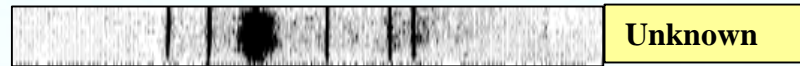
- **RIBOTYPING**, other molecular techniques (rep-PCR, PFGE, RAPD, TGGE, PCR, etc.), Antibiotic Resistance Analysis, F-specific coliphage, Biochemical/nutritional profiles, Chemical & Immunological methods.

# Ribotyping

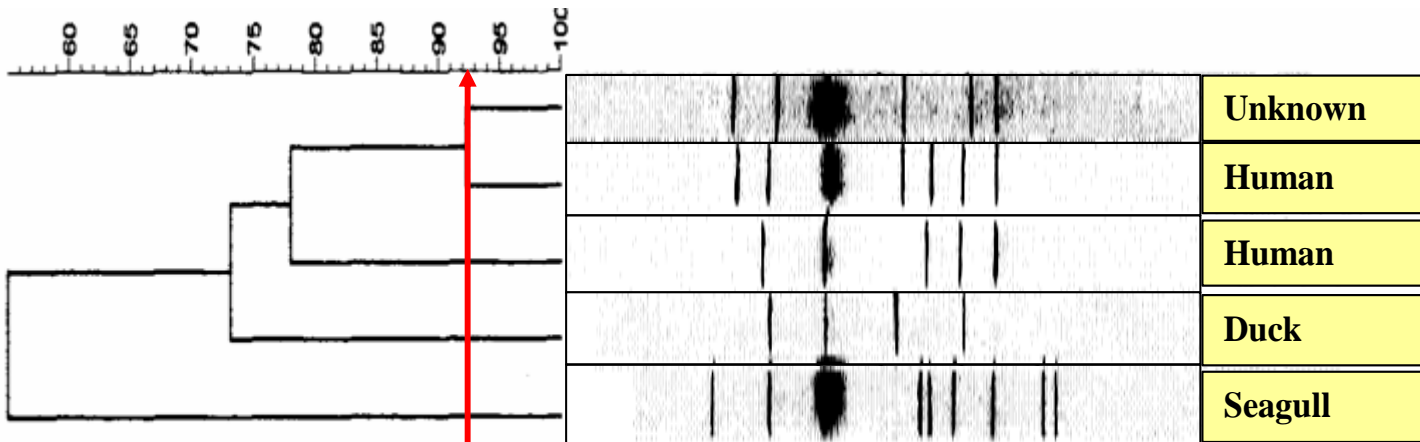
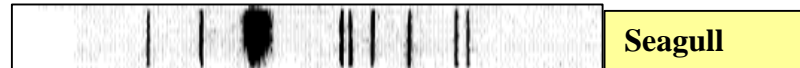
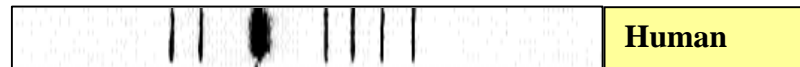
- DNA profiling of bacterial strains;  
Genetically unique intestinal strains in source species:
  - ◆ Adapted to species-specific intestinal environment = unique but consistent mix;
  - ◆ Differ amongst source species.
- Widely accepted for microbial ID.



# Matching ribotype gel banding patterns:



Which of these patterns is most similar to the unknown?



*Human = 93% similarity*

# Ribotyping: *Lab Procedures*

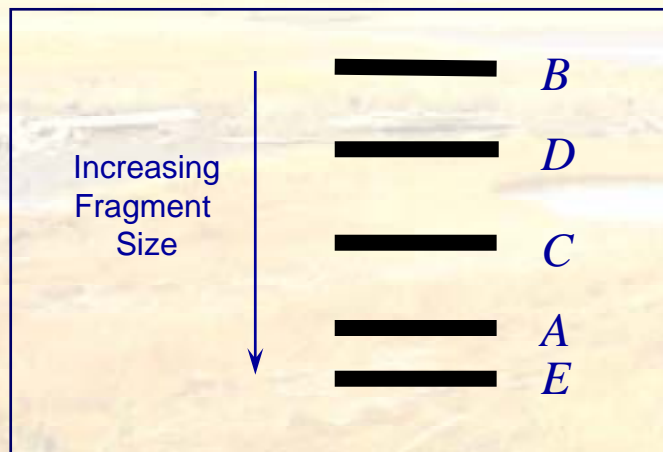
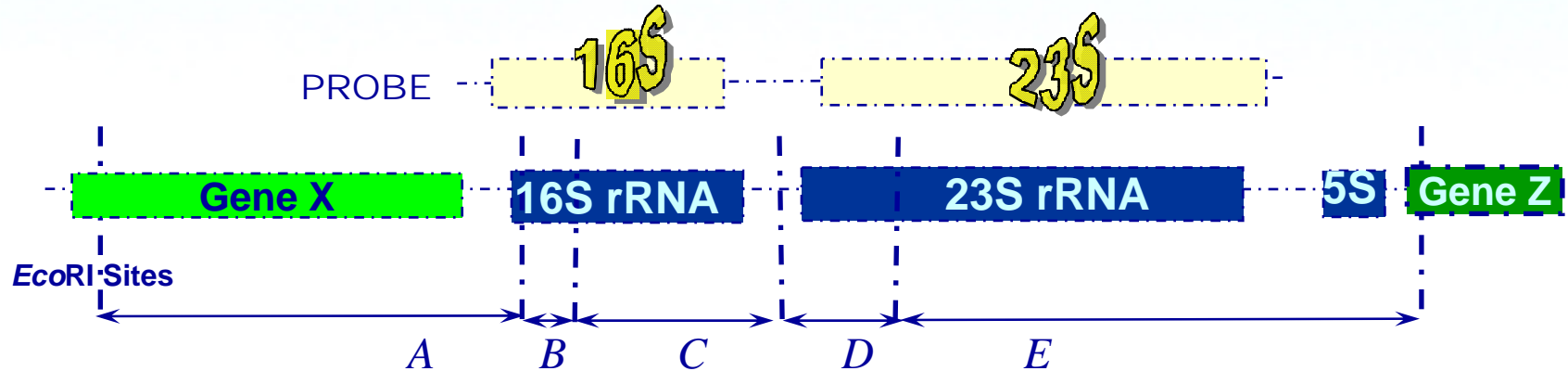
- *E. coli* isolated: source sp. & H<sub>2</sub>O samples.
- DNA extracted & purified.
- DNA digested w/restriction enzyme.
- DNA separated via gel electrophoresis.
- DNA denatured & blotted onto membrane.
- Hybridization with *E. coli* rRNA DNA probe.
- DNA exposed to a chemiluminescent substrate & digitally imaged.
- Image enhanced & optimized in computer.



# RIBOTYPING DNA PROBE

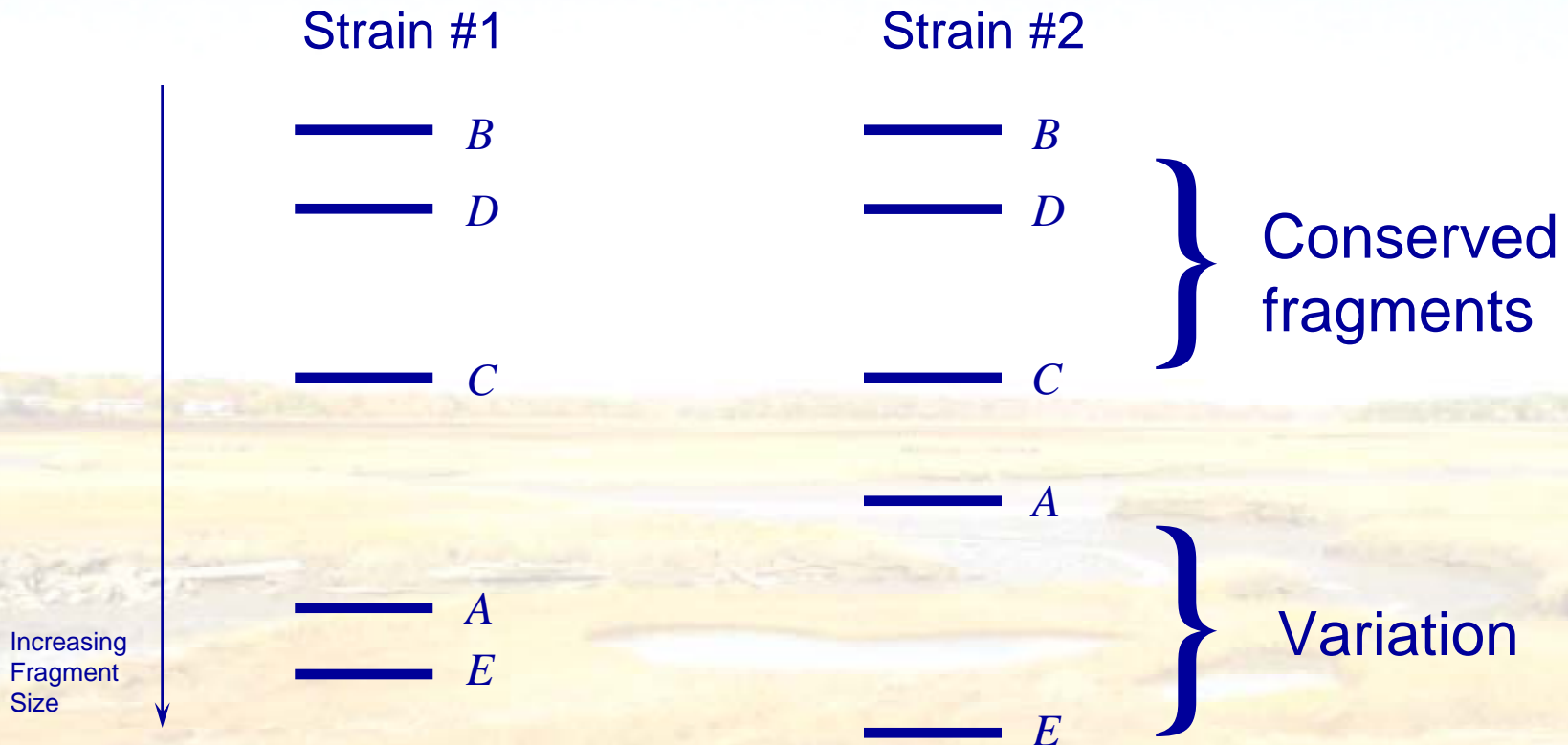
- Derived from *E. coli* rRNA ribosomal operon
- Approximately 6.5 kB
- Contains sequences encoding for:
  - ◆ 16S rRNA
  - ◆ Spacer region including Glu-tRNA
  - ◆ 23S rRNA
  - ◆ 5S rRNA

# Ribotyping: Example Using *EcoRI*



Ribotype pattern generated by *EcoRI* restriction of one ribosomal RNA operon

# Species Differentiation with Ribotyping



*same 16S+ sequence; different ribotype patterns*

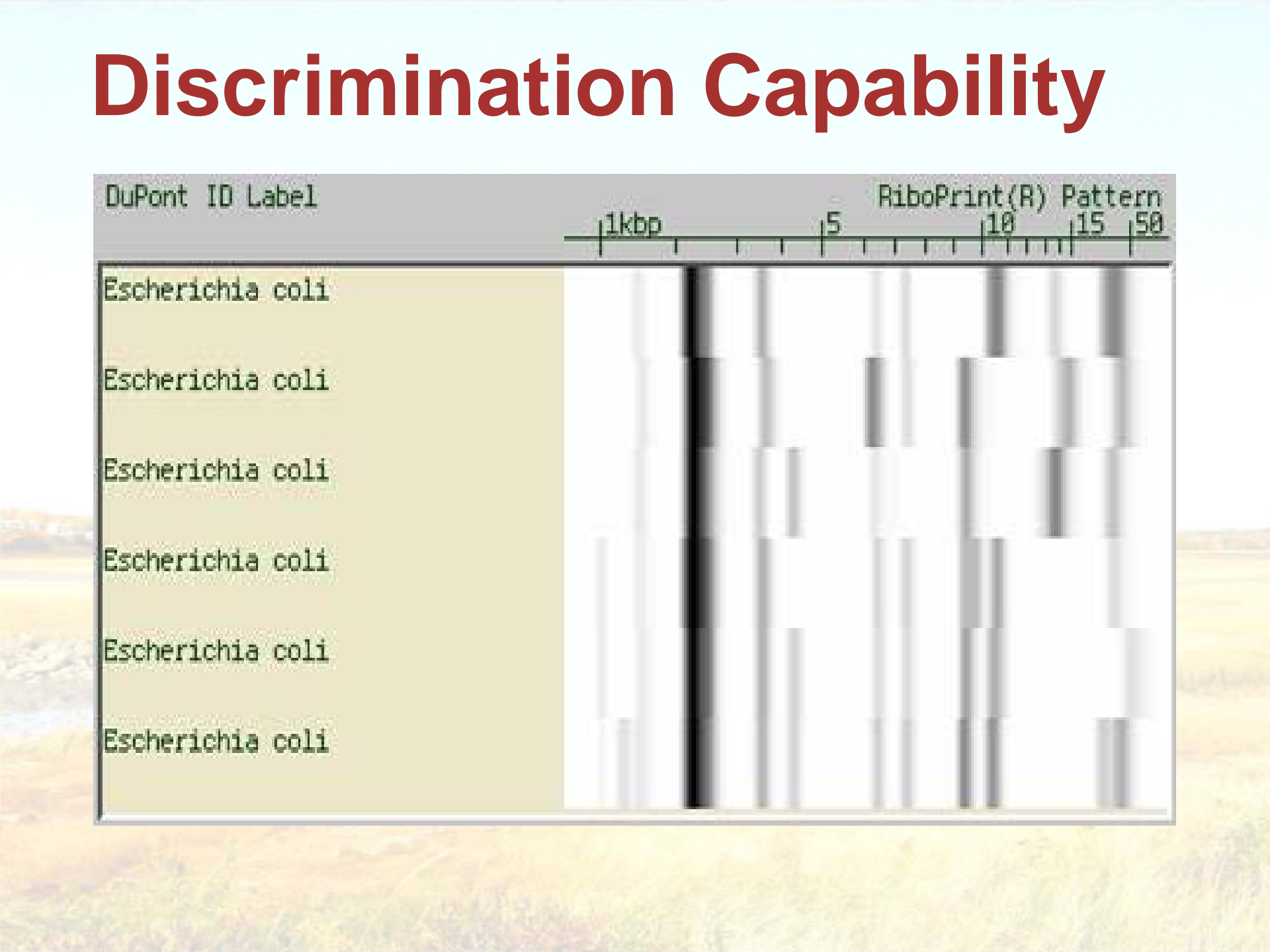


# **Ribotyping:**

## ***Data Analysis***

- DNA patterns are analyzed by cluster analysis and by computing a similarity coefficient.
- Source species identification for sample patterns based on degree of matching to source species patterns.

# Discrimination Capability



# New England Studies (*UNH-ongoing/complete\**)

- Southern ME: (2 studies, 4 watersheds, volunteer monitoring).
- Hampton/Seabrook Harbor, NH.
- Varney Brook & Bellamy River, NH (coastal tributaries; storm events).
- Great Bay, NH (WQ conditions, season, spatial/temporal variation).
- \*Winooski River/ Malletts Bay, VT.

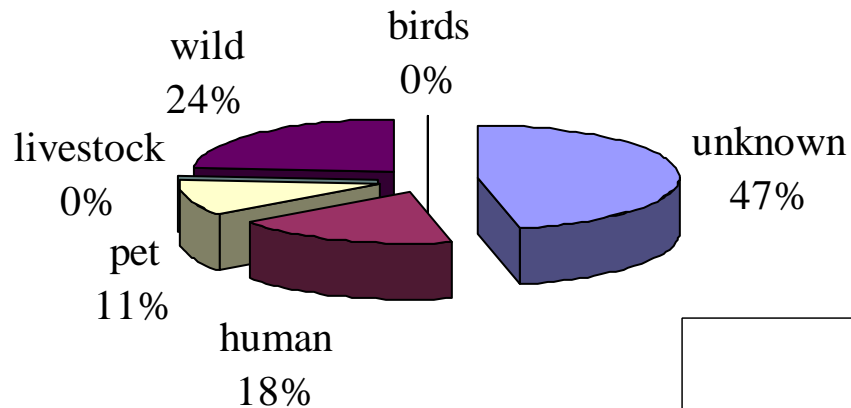


# Wells, ME Source species for identified profiles

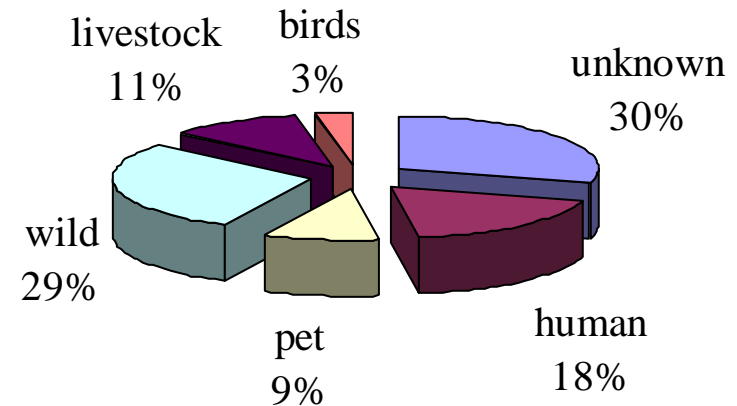
Species	Local Reference Library	Regional Reference Library
<b>Pets</b>		
Cat	-	2
Dog	6	15
<b>Humans</b>		
Stool sample	10	14
Septage	17	17
Wastewater	13	55
<b>Wildlife</b>		
Coyote	10	15
Deer	3	41
Grey Fox	3	3
Muskrat	-	3
Raccoon	4	28
Red Fox	3	26
Squirrel	4	4
<b>Livestock</b>		
Cow	-	30
Horse	-	14
Chicken	-	2
<b>Birds</b>		
Cormorant	-	13
Duck	-	4
Goose	-	19
Grouse	2	2
Pigeon	-	2
Robin	-	3
Seagull	-	5
Total Isolates	75	317
Total Species	11	22

# Summary of MST-Maine Results

**Webhannet Database**



**Regional Database**



# Source Species Databases

Source species	Webhannet	database	Regional	database
type	Isolates	%	Isolates	%
Human	29	18	29	18
Pet	18	11	15	9
Wildlife	38	24	46	29
Birds	0	0	5	4
Livestock	0	0	17	11
Unidentified	74	47	47	30
Total	159		159	



# Percent of *E. coli* isolates identified using 2 databases

	Percent of isolates at different similarity indices			
% Similarity	≥80%	≥85%	≥90%	≥95%
	<i>Webhannet database (1% tolerance)</i>			
% Isolates	41	26	10	2
	<i>NH &amp; ME (2% tolerance)</i>			
% Isolates	82	62	43	18

# Winooski River & Malletts Bay, VT study

- Two adjacent watershed areas next to Lake Champlain, VT.
- Recurrent elevated levels of *E. coli* observed that often exceed water quality standards at numerous sites.
- Numerous potential sources of pollution suspected.
- What are most significant sources at sites of concern?

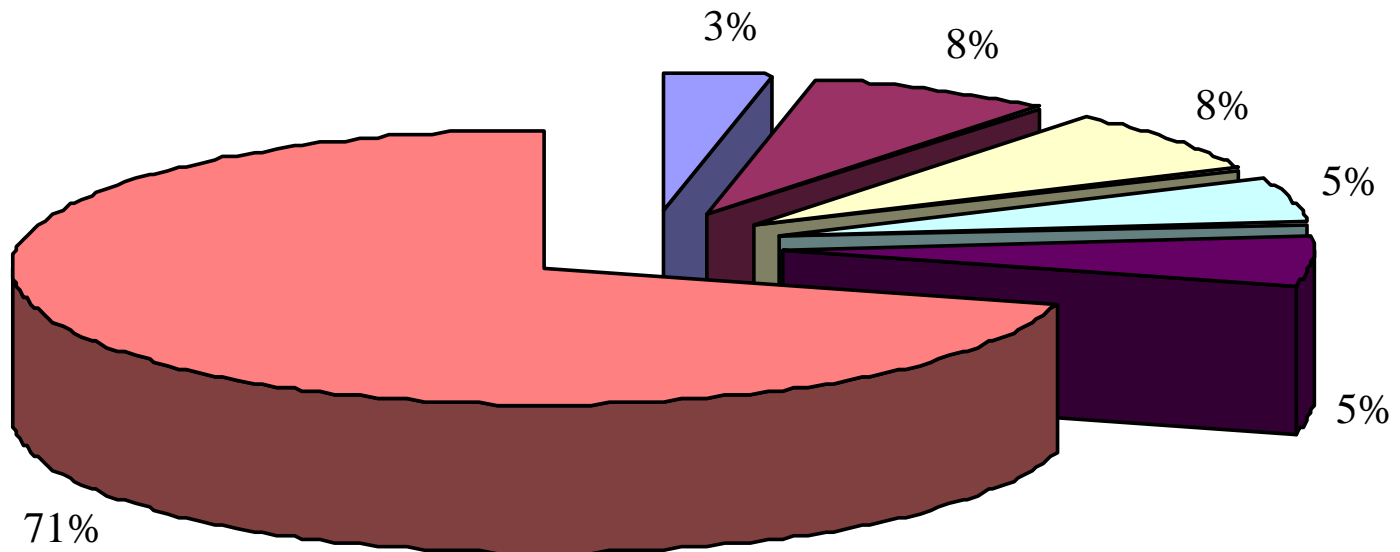
# Source Species Databases: VT, NH & Combined

Species	VT ribotypes	NH ribotypes	VT & NH ribotypes
mallard	17	0	17
cat	18	2	20
cow	29	6	35
dog	10	9	19
seagull	26	5	31
horse	3	14	17
human/septage	16	49	65
pigeon	5	2	7
raccoon	23	14	37
chicken	0	2	2
cormorant	0	14	14
coyote	0	6	6
deer	0	43	43
duck	0	2	2
geese	0	19	19
muskrat	0	5	5
red fox	0	7	7
robin	0	3	3
<b>Totals:</b>	<b>130</b>	<b>202</b>	<b>332</b>



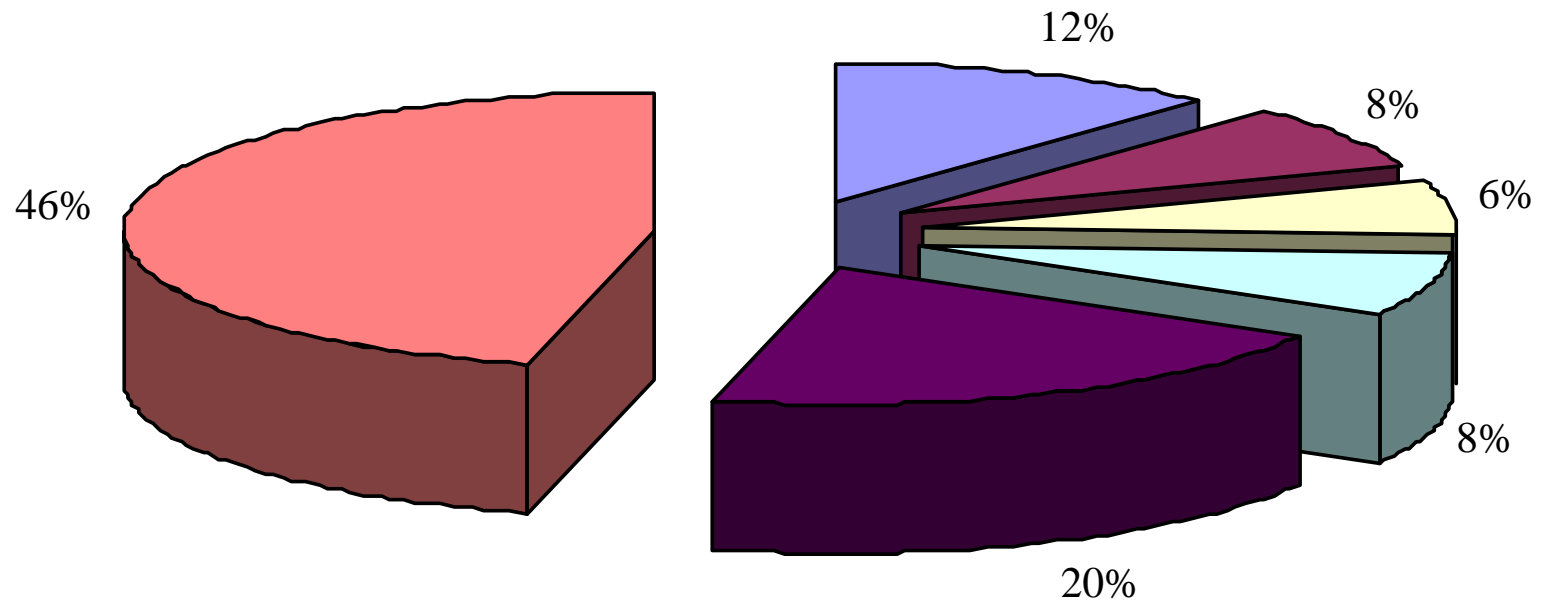
## Using VT Source Species Database

■ Septage ■ Pets ■ Birds ■ Livestock/chickens ■ Wildlife ■ Unknowns



## Using VT + NH Source Species Database

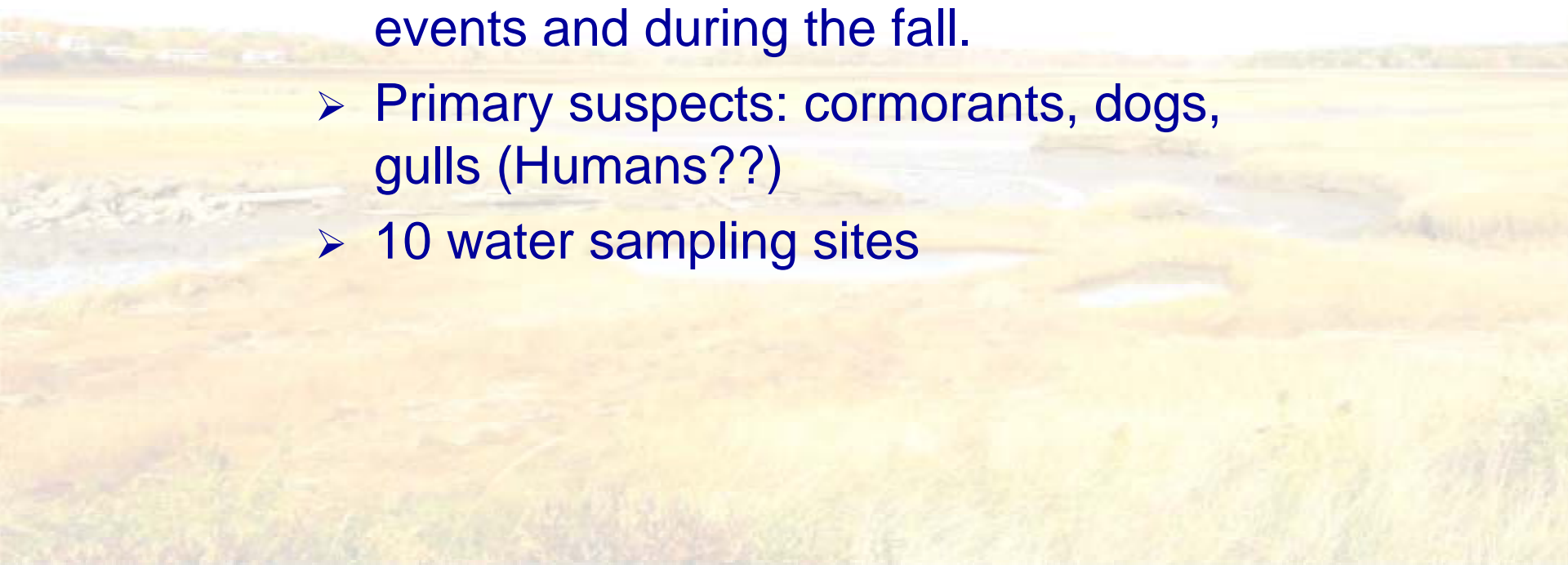
Septage Pets Birds Livestock/chickens Wildlife Unknowns



# Hampton Harbor, NH

## 2000-2001

- Hampton Harbor in Seabrook, Hampton Falls and Hampton, NH
- Important soft shell clam recreational harvesting area closed after rainfall events and during the fall.
- Primary suspects: cormorants, dogs, gulls (Humans??)
- 10 water sampling sites





# Source species for identified profiles

(Hampton Harbor, NH study)

Source species type	# of isolates	% in study area
<b>Septage</b>	<b>102</b>	<b>26%</b>
<b>Pets</b>	<b>15</b>	<b>4%</b>
<b>Birds</b>	<b>29</b>	<b>7%</b>
<b>Livestock/chickens</b>	<b>30</b>	<b>8%</b>
<b>Wildlife</b>	<b>59</b>	<b>15%</b>
<b>Unidentified</b>	<b>155</b>	<b>40%</b>
	<b>390</b>	

# Hampton Harbor study

## *Management Recommendations:*

- Investigate sewage infrastructure for leaking/broken pipes;
- Educate boat owners;
- Investigate remaining septic systems.

# CONCLUSIONS

- Significant fraction of unidentified (unacceptable level of matching) isolates that can be reduced by using a larger database;
- Results for different studies showed a most common source *OR* a mixture with no single dominant source;
- ID'd source species types may or may not fit sample location expectations;
- Water sampling requires clear issue & focus to narrow sites studied and study time period.



# CONCLUSIONS

- *Results provide useful guide for identifying potentially significant sources of fecal contamination.*



# Method Improvement & Research Needs

- A larger, regional source species database would help to improve accuracy & scope of identified source species.
- Temporal and geographical effects.
- Sampling design: sample frequency & study duration; isolates/sample.
- Reduce analytical costs and time.

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